EXTENDED EXPERIMENTAL PROCEDURES

SDS-PAGE and Western Blotting

The proteins in each fraction of the sucrose gradients were TCA-precipitated from 50-400 ul and analyzed by Western blot. Samples were run on 8%, 10% or 4%–12% gradient SDS-polyacrylamide gels and transferred to Optitran BAS-83 membranes (Whatman) by standard methods.

Samples were run on 8% or 4%–12% gradient SDS-polyacrylamide gels and transferred to BAS-33 nitrocellulose membranes (Schleicher and Schuell) by standard methods. Membranes were blocked for 1hr at room temperature in 5% non-fat dry milk (Carnation) in PBS followed by addition of primary antibody for 1 hr at room temperature or overnight at 4 degrees. Blots were washed 1 X 10 min and 5 × 5 min with Western blot wash buffer (23mM Tris, pH 8.0, 190mM NaCl, 0.1% w/v BSA, 1 mM EDTA, 0.5% Triton X-100, 0.02% SDS) after each antibody incubation. HRP-conjugated secondary antibodies (Jackson Immunochemicals) were used at 1:10,000 in 5% milk/PBS for 2 hr at RT, washed as before, and HRP signal was detected by Enhanced ChemiLuminescence (Western Lightning detection kit, Perkin Elmer) and quantified with a Versadoc Imaging System (Bio-Rad) or by densitometry following film scans (Kodak MR film, Quantity One software).

Antibodies

The following antibodies were used throughout the work: anti-FMRP ab17722 (Abcam) at 1:1000, anti-FMRP ab27455 (Abcam) at 1:1000 and HRP-conjugated anti-rabbit (Jackson Immunoresearch) at 1:10,000 for FMRP Western blots; anti-FMRP ab17722, anti-FMRP 2F5 (Gabel et al., 2004), and anti-FMRP 7G1-1 ascites developed by Drs. Ceman and Warren (Brown et al., 2001) was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242, for FMRP immunoprecipitation for CLIP. None of these antibodies crossreacts with family members FXR1P and FXR2P. FXR1P was detected on Western blot using ML13 at 1:20,000 (a gift from Dr. Eduoard Khandjian); FXR2P was detected with 1G2 ascites obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242, used at 1:100. Anti-HuB, C, and D (Hu, neuronal isoforms) and Nova (Ri) patient antisera were used for IPs with IRB institutional approval as previously described (Darnell et al., 2009). Hybridoma cells producing the 2F5 monoclonal were a gift from Justin Fallon (Brown University) and were subcloned, screened by ELISA and used to produce protein G-purified 2F5 IgG by the Rockefeller University Monoclonal Antibody Core Facility.

Anti-PABP rabbit polyclonal antibody ab21060 (Abcam) was used at 1:2000 for Western blots, anti-rpP0 human antibody (US Biological R2031-25) was used at 1:20,000 for Western blot and the indicated concentrations for IP. Anti rpS6 (5G10, 1:1000, Cell Signaling #2217) and anti-rpL5 (ab74744, 1:1000, Abcam) were used for Western blots. Anti-GFP antibody ab6556 (Abcam) was used for immunogold-EM studies. The anti-Hu serum was also used for Western blots at 1:10,000.

Use and Care of Mice

All mice used for these studies were housed in the Rockefeller University Comparative Bioscience Center in accordance with IUCAC-approved protocols for their use and care. We have made every effort to use the minimum number of animals necessary to obtain statistically significant results. Mice were used between the ages of P11 and P30 as indicated, and were bred to yield FVB wild-type and $Fmr1^{tm1Cgr}$ littermate mice. $Fmr1^{tm1Cgr}$ mice (and background-matched FVB controls) were originally obtained from Jackson Labs but bred for many generations in our breeding colony. Similarly, in some cases $Fmr1^{tm1Cgr}$ mice on the C57Bl/6J background (and background-matched C57Bl/6J controls, originally obtained as breeding pairs from Dr. Bill Greenough, U. Illinois, but bred within our colony) were used instead of the FVB background, where noted. For some experiments where WT mice alone were needed CD1 mice were used.

Mouse Brain Polyribosome Preparation

Mouse brain polyribosomes were prepared according to protocols established in our lab (Darnell et al., 2005a; Darnell et al., 2005b; Stefani et al., 2004). Mice of the indicated genotypes and ages were sacrificed by isoflurane anesthesia and decapitation. The brain was removed and placed in ice-cold dissection buffer (1X Hank's balanced salt solution (GIBCO) containing 10 mM HEPES, pH 7.3 (USB) (HHBSS) and 0.1 mg/ml cycloheximide (CHX, Sigma C-7698, made fresh in methanol). Cortex and cerebellum were dissected free of underlying white matter, homogenized in 1 ml polyribosome lysis buffer (10 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mg/ml CHX, 1X Complete EDTA-free protease inhibitor cocktail (Roche), 40 U/ml rRNAsin (Promega)) per brain, with 10-12 strokes at 900 rpm in a motor-driven Teflon-pestle 5 ml glass homogenizer (Wheaton). NP-40 was added to a final concentration of 1% from a 10% stock (Calbiochem) and allowed to sit on ice 10 min. The homogenate was spun at 2,000 x g for 10 min at 4°C. The supernatant (S1) was respun at 20,000 x g for 10 min at 4°C. The resulting supernatant (S2) was loaded onto a 20%–50% w/w linear density gradient of sucrose in gradient buffer (10 mM HEPES-KOH pH 7.4, 150 mM NaCl, and 5 mM $MgCl_2$) prepared using a Gradient Master 107 (BioComp), in 14 × 89 mm polyallomer ultracentrifuge tubes (Beckman 331372). Gradients were centrifuged at 40,000 rpm for 2 hr at 4°C in a Beckman SW41 rotor and sixteen fractions of 0.72 ml volume were collected with continuous monitoring at 254 nm using an ISCO UA-6 UV detector.

Crosslinking-IP Protocol 1

Polyribosome Purification and Pelleting

Polyribosome gradients were prepared from P14, P15 and P25 male FVB wild-type and Fmr1^{tm1Cgr} littermate mice as described above. The S2 supernatants were UV-crosslinked in a 3.5 cm tissue culture dish on a bed of ice-slush three times at 400 mJ/cm^2 (254 nm UV light) with swirling between each irradiation, using a Stratalinker 2400 (Stratagene). Cell-equivalent fractions of these initial purification steps were analyzed by SDS-PAGE and Western blotting for FMRP using ab17722 (Abcam) that does not cross-react with family members FXR1P and FXR2P. Quantitation of three biologic replicates revealed 11.0% (standard deviation 1.4%) of FMRP is present in the P1 fraction. The remaining 90% of brain FMRP in the S2 supernatant was applied to 20%–50% sucrose gradients. The distribution of FMRP in gradient fractions was analyzed by TCA precipitation and SDS-PAGE/Western blot. FMRP sedimented in fractions with a density equivalent to that of the 60S subunit or greater, and the vast majority of it was associated with large polyribosomes. Fractions containing polyribosome-associated FMRP were pooled, diluted 1:1 with gradient buffer and polyribosomes were pelleted by centrifugation for 2 hr at 300,000 x g in polycarbonate centrifuge tubes (Beckman 362305) in a TLA110 rotor (Beckman) using a tabletop ultracentrifuge (Optima MAX, Beckman) at 4°C. SDS-PAGE analysis of the input (S2, pooled polyribosomes) and post-centrifugation supernatant (S3) demonstrated that all the FMRP in these fractions pelleted with polyribosomes (Figure 1D).

Dissociation of RNPs, RNase Treatment, and IP

Polyribosome pellets were dissociated in a 1% SDS-containing buffer based on a published protocol (Hinck et al., 1994) with modifications. The polyribosomal pellet was resuspended in 100 ul SDS-IP buffer (15 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, and 1% SDS), heated to 90 degrees for 10 min with vigorous shaking to dissociate RNP complexes, and diluted with 9 volumes of CSK buffer (20 mM Tris, pH 7.0, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, 300 mM sucrose) and stored on ice. Lysates were treated with 200 units/ml RNase T1 (Applied Biosystems/Ambion AM2280) in CSK buffer for 10 min at 37°C and stopped with the addition of 1320 units of SuperAse-In (Applied Biosystems/Ambion) so that RNAs crosslinked to FMRP were reduced to a modal size of about 100 nucleotides. FMRP was IPed from these samples using Protein A Dynabeads (Invitrogen) bound to either rabbit polyclonal anti-FMRP (ab17722, Abcam) or mouse mixed monoclonal antibodies to 2 different epitopes on FMRP (7G1-1 (Brown et al., 2001) and 2F5 (Gabel et al., 2004)) prepared as described below. We avoided the use of Chemicon ab2160 (also known as 1C3) as it also recognizes the FMRP family paralogs FXR1P and FXR2P. Following IP (described below) the beads were washed sequentially (1 ml washes) with high stringency buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% Na-deoxycholate (DOC), 0.1% SDS, 120 mM NaCl, 25 mM KCl), high salt buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% Na-DOC, 0.1% SDS, 1 M NaCl) and twice with low salt buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA) followed by one wash with NT-2 buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, and 0.05% NP-40). After each capture on the magnet beads, the beads were resuspended by end-over-end rotation for a few minutes. Resuspended beads were transferred to new microfuge tubes once during the protocol, and throughout the washes tubes were treated in a random order to diminish the chance of artifacts. Throughout the CLIP protocol 1.5 ml SlickSeal microfuge tubes (National Diagnostics) were used to reduce non-crosslinked RNA binding to tubes.

Dephosphorylation of RNA, 3′ Linker Ligation, and SDS-PAGE Separation of RNABP:RNA Complexes

Immunoprecipitations were treated with calf intestinal phosphatase to remove the 5′ phosphate from RNA crosslinked to FMRP (''RNA tags'') so that the RNA could not circularize during ligation, an otherwise predominant competing intramolecular reaction. Beads were resuspended in 0.08 ml of 1X dephosphorylation buffer and 3 units of calf intestinal alkaline phosphatase (CIAP, Roche) was added, incubated in the Thermomixer R at 37°C for 20 min, programmed to shake at 1000rpm for 15 s every 4 min, followed by 3X 1ml washes with NT-2, 2X with 1 ml PXL (1X PBS (tissue culture grade; no Mg^{+2}, no Ca^{+2}, GIBCO), 0.1% SDS, 0.5% Na-DOC, 0.5% NP-40), and 2X with NT-2 with one tube change during washing. To ligate a P^{32}-labeled RNA linker to the 3′ end of the RNA, the puromycin-blocked linker (L32, 5′-OH-GUGUCAGUCACUUCCAGCGG-3′-puromycin, Dharmacon) was first labeled using T4 phosphonucleotide kinase (PNK, New England Biolabs, NEB). 50 pmol of L32 RNA, 0.015 ml P^{32}-γ-ATP, and 2 ul of T4 PNK in 1X PNK buffer were incubated at 37°C for 30 min, an additional 0.02 ml 10 mM ATP was added and the reaction incubated 5 min. Radiolabeled linker was spun through a G-25 column (Amersham) to remove free ATP. 30 pmol of the labeled 3′ RNA linker was added to a 0.08 ml T4 RNA ligase reaction (Fermentas), according to kit instructions, and on-bead ligation reactions were incubated at 16°C for 1 hr in the Thermomixer R programmed 1000rpm for 15 s every 4 min. After 1 hr, 80 pmol of L32 RNA linker WITH 5′ phosphate was added to each tube of reaction and incubated overnight followed by 3X wash with NT-2 buffer. To rephosphorylate the 5′ end of the RNA for 5′ linker ligation, 80 ul of T4 PNK mix (6 ul of 1 mM ATP, 4 ul T4 PNK enzyme (NEB), 1 ul RNasin in 1X T4 PNK buffer (NEB), total volume 80ul) was added to each tube and incubated for 20 min at 1000rpm shaking for 15 s every 4 min and washed 1X with PXL and 2X with NT-2 buffers. Each tube of beads was resuspended in 40 ul NuPAGE loading buffer (LDS, Invitrogen), containing reducing agent, and incubated at 70°C for 10min at 1000rpm. Supernatants were taken off the beads and run on Novex NuPAGE 10% Bis-Tris gels (Invitrogen) in MOPS running buffer (Invitrogen) for 3 hr at 175V and transferred to Protran nitrocellulose (S&S) using a Novex wet transfer apparatus (Invitrogen). After transfer, the nitrocellulose was quickly rinsed with RNase-free PBS, blotted with Kimwipes, wrapped in plastic wrap and exposed to Biomax MR film (Kodak).

Recovery of Complexes, Protease Digestion, and 5′ Linker Ligation

Nitrocellulose membranes were aligned carefully with the exposed film and filter excised with a scalpel from 105-140 kDa kDa (the size range of FMRP crosslinked to RNA of approx. 100 nucleotides). Each band of nitrocellulose membrane was further cut into

smaller pieces and proteinase K treated (0.2 ml of a 1:5 dilution of proteinase K (4 mg/ml, Roche) in PK buffer (100 mM Tris-Cl pH 7.5, 50 mM NaCl, 10 mM EDTA)) at 37°C, 1100 rpm for 20 min. Then 0.2 ml of PK+7M urea solution was added and incubated for another 20min at 37°C at 1100 rpm. Finally, 0.4 ml of RNA phenol (pH 6.8, Applied Biosystems/Ambion) and 0.13 ml of 49:1 $CHCl_3$:isoamyl alcohol were added and incubated at 37°C, 1100 rpm for additional 20 min. Tubes were spun at 20,000 x g in a desktop microcentrifuge. Glycogen (0.5 ul, Applied Biosystems/Ambion), 50 ul 3M NaOAc pH 5.2, and 1 ml of 1:1 ethanol:isopropanol were added to the aqueous phase in a fresh tube and RNA was precipitated overnight at −20°C. RNA was pelleted, washed with 75% ethanol, the pellet dried and dissolved in 6 ul RNase-free H_2O. RNA ligation to add the 5′ linker (L51 (RNA linker 51), 5′-OH-AGGGAGGAC GAUGCGG-3′) was performed with 1 ul 10X T4 RNA ligase buffer (Fermentas), 1 ul BSA (0.2ug/ul), 1 ul ATP (10mM), 0.1 ul T4 RNA ligase (3U, Fermentas), and 20 pmol of L51 RNA linker in a total volume of 10 ul at 16°C overnight. To the ligation reaction, 77 ul H_2O, 11 ul 10X RQ1 DNase buffer, 5 ul RQ1 DNase (Promega) and 5 ul RNasin (Promega) were added and incubated at 37°C for 20 min. 0.3 ml H_2O, 0.3 ml RNA phenol (Ambion) and 0.1 ml $CHCl_3$ were added, vortexed, spun and the aqueous layer taken. RNA was precipitated with 50 ul 3M NaOAc pH 5.2, 1 ul glycogen (Ambion) and 1 ml 1:1 ethanol:isopropanol overnight at −20°C.

RT-PCR and High-Throughput Sequencing of PCR Products

RNA was pelleted, washed, dried, and resuspended in 8 ul of RNase-free H_2O. The RNA was mixed with 2 ul of primer P32 (3′ DNA primer 32, 5′- CTTCACTCACCTCGCAACCG-3′, Operon) at 5 pmol/ul, incubated at 65°C for 5 min, chilled, and spun. 3 ul 3mM dNTPs, 1 ul 0.1M DTT, 4 ul 5X SuperScript RT buffer, 1 ul RNasin, and 1 ul SuperScript III (Invitrogen) were added and incubated at 50°C for 30 min, 90°C for 5 min and briefly at 4°C. PCR reactions were performed with 27 ul Accuprime Pfx Supermix (Invitrogen), 1 ul P51 (5′ DNA primer 51, 5′- AGGGAGGACGATGCGG-3′, Operon) at 5 pmol/ul, 1 ul P32 primer, 5 pmol/ul and 2 ul of the RT reaction, cycled for 20-25 cycles using a program of 95°C for 20 s, 61°C for 30 s, and 68°C for 20 s. A 10% denaturing polyacrylamide was poured and the entire PCR reaction was loaded along with 3 ul of Amplisize Molecular Ruler (Biorad). To visualize DNA, the gel was immersed in a 1:10,000-fold dilution of SYBR Gold (Molecular Probes) in TBE for 10 min. Routinely, aliquots of the reaction were removed at various cycle numbers (Figure 1F, 34-38 cycles) and product excised from the lowest cycle number giving visible product. Acrylamide bands containing DNA of 60-100 nts were excised and DNA purified using a QIAGEN kit (protocol for polyacrylamide gel) and resuspended in 15 ul of H_2O. An additional PCR reaction was performed using the following fusion primers. AP5fusion1: 5′-GCCTCCCTCGCGCCATCAGCG*AGGGAGGACGATGCGG*-3′; AP5fusion2: 5′-GCCTCCCTCGCGCCATCAGAC*AGGGAGGACG ATGCGG*-3′; AP5fusion3: 5′-GCCTCCCTCGCGCCATCAGTA*AGGGAGGACGATGCGG*-3′; AP5fusion4: 5′-GCCTCCCTCGCGCCA TCAGCT*AGGGAGGACGATGCGG*-3; AP5fusion5: 5′-GCCTCCCTCGCGCCATCAGGC*AGGGAGGACGATGCGG*-3′; AP5fusion6: 5′-GCCTCCCTCGCGCCATCAGGA*AGGGAGGACGATGCGG*-3′.

From the 5′ end the primers begin with the 454 Life Sciences 'Adaptor A' followed by a unique 'di-tag' (underlined), providing the ability to sequence multiple experiments simultaneously, and finally, in bold is the sequence matching the 5′ RNA linker (L51) used. One 3′ fusion primer was designed consisting of a sequence matching the 3′ RNA linker used (italicized) and that of the 454 Life Sciences 'Adaptor B': BP3fusion: 5′-GCCTTGCCAGCCCGCTCAG*CCGCTGGAAGTGACTGACAC*-3′.

PCR re-amplification was performed using Accuprime Pfx (Invitrogen) and ranged between 6 and 15 cycles. The product was then run on a 2% agarose gel and purified using Qiaex II beads (QIAGEN). A total of 100 ng of DNA was submitted for sequencing per run. The sequencing of CLIP tags was performed using 454 Life Sciences Adaptor A as the sequencing primer.

Crosslinking-IP Protocol 2

Protocol 2 was carried out with the same basic design as in Protocol 1 with the following changes. Male CD1 mice, P11-P13, were used, and tissue was rapidly sliced with scalpels into approx. 1 mm slices in ice-cold HHBSS before crosslinking, which was carried out as quickly as possible after sacrifice. The S2 supernatant (1 ml) was layered onto a 2 ml cushion of 20% sucrose in gradient buffer and spun for 2 hr at 300,000 x g in a TLA110 rotor in an Optima MAX centrifuge (Beckman). The resulting polyribosomal pellet was resuspended in SDS-IP buffer containing 2% SDS and incubated at 70°C for 10 min with vigorous shaking. 19 volumes of CSK buffer were added to dilute the SDS to 0.1% for RNase digestion and IP. RNase Cocktail, a mixture of RNases A and T1 for more complete digestion (AM2286, Applied Biosystems/Ambion), was added to aliquots of the resuspension, each representing 1/3rd of a mouse brain, using dilutions ranging from no added RNase to a dilution of 1:100,000, and bands excised from the nitrocellulose were chosen based on migration (indicated with brackets in Figure 1G-H). The RNase cocktail was stopped with a mixture of SuperAse-In (Applied Biosystems/Ambion) and RNAsin (Promega) which varied depending on amount of cocktail added. Following RNase digestion the sample was split in two and IPed with both anti-FMRP (ab17722-loaded beads) or anti-Hu loaded beads (see section on IP). FMRP and Hu IPs were treated identically throughout the protocol except for the SDS-PAGE separation on Novex NuPAGE gels. No reducing agent was added to the Hu samples to keep the IgG intact so that immunoglobulin heavy chain would not interfere with migration of Hu:RNA complexes at 55-65 kDa. During the 3′ linker ligation unlabelled L32 was used as follows: 40 ul linker mix was prepared from 8 ul 3′ linker (5′-phospho-L32) at 20 pmol/ul and 32 ul water and added to beads followed by 40 ul of ligase mix (14 ul water, 8 ul 10X T4 RNA ligase buffer, 8 ul BSA, 8 ul 10 mM ATP, 2 ul T4 RNA ligase (Fermentas)) and ligated overnight at 16°C shaking at 1000 rpm every 4 min for 15 s in a Thermomixer R. Beads were washed 3X with PNK buffer (50mM Tris-Cl pH 7.4, 10mM $MgCl_2$, 0.5% NP-40), and then RNA was 5′ end-labeled on the beads using T4 PNK as described with the addition of 2 ul of P^{32}-γ-ATP in the 80 ul labeling mix for 20 min at 37°C with shaking. 10 μl of 1 mM ATP was added and the reaction incubated an additional 5 min. Beads were then washed 3X with PNK/EGTA buffer (50mM Tris-Cl pH 7.4, 20mM EGTA, 0.5% NP-40). For the 5′ linker ligation, a degenerate 5′ RNA linker (RL5D) was used to distinguish between identical RNA tags that arise in the dataset because of PCR

amplification (they will have the same degenerate code in the linker) versus separately crosslinked tags that happen to be identical (they will have different degenerate codes). RL5D: 5′-OH-AGGGAGGACGAUGCGGr(N)r(N)r(N)r(N)G3′-OH.

Following an initial RT-PCR reaction using DNA primers complementary to RNA linkers an additional PCR reaction was performed using the following fusion primers to permit sequencing on the Illumina platform. SP5fusion: 5′AATGATACGGCGACCACC GA*CTATGGATACTTAGTC*AGGGAGGACGATGCGG-3′.

Underlined is the sequence complementary to the 5′ RNA linker used. In italics is the sequence to which the 'custom' sequencing primer will anneal. The remaining sequence at the 5′ end is that of the Illumina adaptor sequence "P5." SP3fusion: 5′CAAGCAGAAGACGGCATACGACCGCTGGAAGTGACTGACAC3′.

Underlined is the sequence complementary to the 3′ RNA linker used. The remaining sequence at the 5′ end is that of the Illumina adaptor sequence "P7."

PCR amplification was performed using Accuprime Pfx (Invitrogen) and ranged between 8 and 14 cycles. The product was then run on a 2% agarose gel and purified using Qiaquick spin columns (QIAGEN). A total of ~10-30 μl of DNA (quantified using the Quant-IT kit (Invitrogen)) at 10 nM was submitted for sequencing. The sequencing of CLIP tags was performed using the sequencing primer SSP1: 5′-CTATGGATACTTAGTCAGGGAGGACGATGCGG-3′.

The sequencing primer is a custom sequence designed based on our 5′ RNA linker sequence, taking into consideration the characteristics needed to function as a Illumina sequencing primer (e.g., length, Tm, etc.) This was necessary in order to begin reading sequence directly following the base linker/primer sequence (degenerate code and CLIP tag only).

FMRP CLIP on Steady-State and Stalled Complexes

Three WT FVB mice (P24) were used for the standard puromycin run-off in the IVTEBP system as described in a following method. The S1 post nuclear lysate from three mice was 300 ul to which was added ATP to 2 mM, mixed amino acids (Promega) to 0.1 mM and 150 ul of nuclease-treated rabbit reticulocyte lysate (RRL, Promega). The reaction was divided into 2 equal parts and CHX added to one (0.1 mM final conc.) and puromycin (7.2 mg/ml) to the other. After 20 min of run-off at 30°C, the reactions were terminated by the addition of 1 ml of lysis buffer containing CHX (10 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mg/ml CHX, 1X Complete EDTA-free protease inhibitor cocktail (Roche), 40 U/ml rRNAsin (Promega) and NP-40 added to a final concentration of 1%. After 10 min on ice, samples were crosslinked once at 400 mJ/cm^2 (Stratalinker 2400) in a 3.5 cm dish on a bed of ice-slush, before centrifugation at 20,000 x g for 10 min and purification of the supernatant on a 20%–50% linear sucrose gradient. FMRP Western blot (ab17722) on TCA-precipitated samples from each fraction was used to identify FMRP-containing fractions. Fractions 9-12 (CHX gradient) or 6-9 (puromycin run-off grandient) were pooled, diluted 1:1 with gradient buffer and complexes spun for 3 hr at 300,000 x g in a TLA110 rotor in an Optima MAX centrifuge (Beckman). Pelleting of all the FMRP was confirmed by FMRP Western blot of the input and the post-spin supernatant as in Figure 1D. The resulting polyribosomal pellet was treated as in Protocol 2 including one wash with gradient buffer, resuspension in SDS-IP buffer containing 2% SDS, and incubation at 70°C for 10 min with vigorous shaking. 19 volumes of CSK buffer were added to dilute the SDS to 0.1% for RNase digestion and IP. From this point on CLIP was conducted exactly as in Protocol 2, using ab17722-loaded Protein A Sepharose Dynabeads for the FMRP IPs.

An independent biological replicate of this experiment was performed as a positive control and is referred to as Expt. 2 in Table S7.

Immunoprecipitations for CLIP

FMRP

0.2 ml of antibody loaded-beads were prepared using Protein A Dynabeads washed 3X with 0.1 M Na-phosphate buffer, pH 8.1, plus 0.01% Tween-20 (bead wash buffer, BWB) and resuspended in 0.2 ml BWB after the final wash. For polyclonal antibody IPs 0.13 mg IgG of ab17722 (Abcam) was added to 0.2 ml washed beads, rotated in the cold 1 hr at 4°C, followed by 3X wash with BWB (1 ml each time) and resuspension in 0.2 ml BWB. Beads were stored on ice or at 4°C until use. This amount of beads was used to IP one mouse brain equivalent of FMRP. For mixed monoclonal IPs (we use mixed, yet specific, monoclonals to markedly increase avidity) 0.2 ml washed beads were rotated with 0.1 ml of rabbit anti-mouse IgG (2.3 mg/ml IgG, Jackson Immunochemicals 300-800) for 30 min at RT, washed 3X with BWB and then bound to a mixture of 0.288 mg IgG from 7G1-1 ascites at 6 mg/ml IgG (Univ. of Iowa Hybridoma Bank) and 0.288 mg IgG from 2F5 (protein G purified from bioreactor supernatant, the Rockefeller University Monoclonal Facility, see antibody section), then washed and stored in 0.2 ml as described for polyclonal beads. The rabbit anti-mouse bridging antibody was used to increase retention of the mouse antibodies on protein A beads through very stringent washes and the lengthy CLIP protocol. This is not necessary for rabbit or human IgG.

Hu

beads for Hu IPs were prepared in the same way, using 0.04 ml of human paraneoplastic anti-Hu antiserum per 0.2 ml washed protein A Dynabeads.

The indicated volume of resuspended, antibody-loaded beads were added to prepared lysates and IPs were performed for 2-4 hr at 4°C with end-over-end rotation in a cold room. Beads were captured on a magnet (Invitrogen) and washed as described in each CLIP protocol. In general, aliquots of the prepared lysate and the post-IP supernatant were compared by Western blot for depletion of FMRP or Hu to confirm that the IP was successful.

Immunoprecipitation of rpP0 from the Puromycin- and MN-Resistant Complex

The ribosome and associated proteins in the same complex were co-IPed using human anti-rpPO and anti-Nova human autoantisera as a negative control. Beads were prepared as for CLIP using 60 ul of anti-P0 and 60 ul of anti-Nova per 160 ul of washed Protein A Dynabeads followed by 3 additional washes in BWB and resuspension to 160 ul in BWB. Preclearing beads were prepared by loading 80 ul of washed beads with 30 ul of rabbit anti-mouse IgG (Jackson ImmunoResearch). Input to the IP was prepared from the cortex and cerebellum of 2 WT and 2 *Fmr1* KO mice (P14-P18, FVB) exactly according to the protocol described in the "IVTEBP system and ribosomal run-off assays" method below. Following puromycin run-off at 30°C for 20 min, and addition of 1 ml of lysis buffer containing CHX to stop the reaction, NP-40 was added to a final concentration of 1%, incubated on ice for 10 min and Ca^{+2} was added to a concentration of 1 mM. Micrococcal nuclease (1000 U/ml final concentration, NEB) was added to both samples and incubated at room temperature for 20 min with end-over-end mixing. The reaction was stopped with the addition of 2 mM EGTA, lysates spun at 20,000 x g for 10 min (4°C), and supernatants fractionated on linear 20%–50% sucrose gradients as described above. Fractions containing FMRP (by TCA precipitation and Western blot) were pooled and diluted 1:1 with gradient buffer. WT and KO lysates were aliquotted at 1.2 ml per microfuge tube and precleared with 8 ul of preclearing beads for 1 hr at 4°C with end-over-end mixing. Precleared supernatants were pooled by genotype and redistributed as 1.3 ml aliquots into each of two tubes per genotype. 50 ul of anti-rpP0 or anti-Nova beads were added to each tube and IPed for 2 hr at 4°C with end-over-end mixing. Following bead capture on the magnet, beads were washed 4 times with 1 ml of gradient buffer containing 1% NP-40. After the final capture the beads were eluted with 30 ul of NuPAGE loading buffer (LDS, Invitrogen), containing reducing agent, by incubation at 70°C for 10 min at 1000 rpm. Supernatants were removed from the beads and run on Novex NuPAGE 10% Bis-Tris gels (Invitrogen) in MOPS running buffer (Invitrogen) and transferred to BAS-83 nitrocellulose (S&S) using a Novex wet transfer apparatus (Invitrogen). Western blots were performed as above, using anti-FMRP ab27455, anti-rpS6 and anti-rpL5 to probe for proteins co-immunoprecipitating with rpP0 (a component of the 60S ribosomal subunit).

Determination of the Relative Abundance of mRNAs in Pooled Polysome Fractions

Total RNA from polysome gradients was extracted by using TRIzol LS (Invitrogen) according to manufacturer's instructions and was purified again using RNeasy Mini kits including the DNase I digestion step (QIAGEN). Probes were made by the Whole Transcript Sense Target Labeling Assay (Affymetrix) according to instructions and applied to Mouse Exon 1.0 ST Array chips (Affymetrix) by the Rockefeller University Genomics Resource Center. Gene expression levels were summarized from extended probe sets by the IterPLIER model using Affymetrix power tools (APT, Affymetrix). A pseudo count of 8 was added to the estimated intensity for each gene before $\log_2$ transformation.

Definition of a Neuronal mRNA Population for Use as Background in GO, Ingenuity Pathway Analysis, and Comparison with other Data Sets

Processed gene expression intensities in neuronal cells were downloaded from GEO (GSE9566) (Cahoy et al., 2008)). Only genes with $\log_2$ intensity $\geq$ 7 were kept for analysis.

Annotation of High-Throughput Sequencing Data

Mapping of CLIP Reads to the Mouse Genome

454 reads collected from five different FMRP CLIP experiments were mapped to the mouse genome (NCBI37/mm9, July 2007) by BLAT (Kent, 2002), using the parameters stepSize = 5 and minScore = 15. A read whose best score was higher than the second best was considered to be unambiguously mappable. Reads without unambiguous mapping were excluded from further analysis. For each of the five protocol 1 CLIP experiments in which the full-length of the RNA tag was sequenced, reads with the same start and stop positions were considered to be duplicates potentially resulting from RT-PCR amplification, and collapsed to identify unique reads used for further analysis.

CLIP protocol 2 was used to perform two additional FMRP CLIP experiments and two Hu CLIP experiments and used the Illumina Genome Analyzer to obtain 76-nucleotide (nt) reads, including a 5-nt degenerate "barcoded" linker sequence read-able from the 5′ end of each CLIP tag to distinguish potential PCR duplicates from genuinely unique reads mapped to the same locations. Therefore, the sequence of actual RNA tags was determined up to 71 nt in length, and the relatively long reads maximized the chance of detecting the full-length RNA fragments (modal size ~50 nt) pulled down by immunoprecipitation. To minimize sequencing errors resulting from the ultra-high-throughput sequencing and hence alignment errors in downstream analysis, we first filtered the raw reads based on the quality scores. We required a minimum quality score $\geq$ 20 in the first five nucleotides (the degenerate region), and an average score $\geq$ 20 in the next 25 nucleotides (i.e., the first 25 nucleotides of each actual RNA tag). The filtered reads were then subject to the removal of the 5′ degenerate "barcode" (whose identity was however recorded) and the 3′ linker to get the actual RNA tag sequences. Since RNA CLIP tags differ in size, 3′ linkers were detected and trimmed by aligning each read to the linker sequence using the needle program (parameters: gapopen: 100, gapext 10 for alignment; #matches-#mismatches $\geq$ 4 nt, %mismatches $\leq$ 15%, and #indels $\leq$ 1 nt for detection of the linker (Rice et al., 2000)). The linker-trimmed reads were then mapped to the mouse genome (NCBI37/mm9, July 2007) by the program Novoalign (http://www.novocraft.com), requiring $\leq$ 2 mismatches (substitutions or indels) in $\geq$ 28 nt. Only reads with unambiguous mapping were kept. Mappable reads for all four experiments were then pooled together, so that PCR duplicates and potential contaminations (e.g., between neighboring lanes in the flow cells during Illumina

sequencing) were eliminated to identify unique reads, using an expectation-maximization (EM)-based algorithm, which modeled the starting coordinates of mappable tags, and the identity and copy number of their degenerate "barcode" sequences (described in details below).

Identifying Unique CLIP Tags in the Presence of a Random Barcode Sequence

Illumina CLIP tags with the same starting positions are frequently duplicates derived from RT-PCR amplification, and thus should be collapsed. However, in some cases they represent independent protein-RNA interactions. Ideally, genuinely unique CLIP tags can be distinguished from RT-PCR duplicates in the presence of distinct degenerate barcode sequences, which were attached to the 5′ end of CLIP tags before PCR. In practice, the process is complicated by sequencing errors in the barcode, so that different barcodes do not guarantee their uniqueness for tags with the same starting genomic coordinates. This is especially true when a high number of PCR cycles are applied. For instance, for a CLIP tag with 1,000 copies and at the 1% sequencing error rate (which is typical for the Illumina platform (Wang et al., 2008)), ~5% or 50 tags are expected to have $\geq$ 1 sequencing errors in the barcode. Therefore, methods relying on different barcode sequences in a naive way will report ~50 unique tags, among which only one is bona fide. In other words, the barcode sequences of unique tags should be "sufficiently" more distinct than one would expect from sequencing errors. To address this concern, we used an EM algorithm to infer unique reads by modeling the identity and copy number of the barcode sequences for reads starting at each particular genomic position.

More formally, we denote the RNA fragments in the CLIP library as "tags," and the recovered tags by sequencing as "reads." Read sequences might be different from tag sequences due to sequencing errors. We assume that each of the K genuinely unique tags with a particular genomic starting coordinate have a different barcode sequence, which is denoted as s_k (k = 1,2,…,K). This assumption is reasonable because the complexity of a CLIP library is relatively limited due to stringent selection and purification. After PCR amplification, the relative abundance of each tag in the library is denoted as $\alpha_k, \sum_{k=1}^{K} a_k = 1$. After sequencing, denote the observed barcode in each of the N reads successfully mapped to the correct genomic position as o_i (i = 1,2,…,N). The likelihood of observing a particular barcode sequence in read i, given the identity and abundance of the each tag, can be written as

$$P(o_i|\Theta) = \sum_{k=1}^{K} P(o_i|s_k)a_k, \tag{1}$$

where Θ is the collection of all model parameters. The probability of getting an observed o_i from an actual tag s_k can be determined by the sequencing error rate and their editing distance (number of substitutions because we ignored indels in barcode here):

$$P(o_i|s_k) = e^{|o_i - s_k|}, \tag{2}$$

where e is the sequencing error at the nucleotide level and $|o_i - s_k|$ is the editing distance. For Illumina sequencing, it was estimated to be 1%–2% (Wang et al., 2008).

The likelihood of observing all reads mapped to the position can thus be written as

$$L(\Theta) = \prod_{i=1}^{N} P(o_i|\Theta) = \prod_{i=1}^{N} \left\{ \sum_{k=1}^{K} P(o_i|s_k)a_k \right\} \tag{3}$$

The parameter α_k can be solved iteratively by a standard EM algorithm by maximizing likelihood, as shown below.

E-step: estimate the posterior probability that the read o_i is actually derived from the tag s_k, and α_k.

$$P(s_k|o_i) = \frac{P(o_i|s_k)a_k}{\sum_{l=1}^{K} P(o_i|s_l)a_l} \tag{4}$$

$$\widehat{a}_k = \frac{\sum_{i=1}^{N} P(s_k|o_i)}{N} \tag{5}$$

M-step: update the nucleotide-level sequencing error rate.

$$\widehat{e} = \frac{1}{NL} \sum_{i=1}^{N} \sum_{k=1}^{K} P(s_k|o_i)|o_i - s_k| \tag{6}$$

where L = 5 is the length of each barcode and e is bounded in the range between 0.001 and 0.02. After the EM procedure converges, the final estimates of $P(s_k \mid o_i)$ and $\alpha_{k.}$ are obtained. We can then estimate the probability that at least one read is actually derived from the tag s_k as a measure of the confidence of s_k being actually present in the CLIP library

$$1 - \prod_{i=1}^{N}\{1 - P(s_k|o_i)\} \quad (7)$$

As a convention, we report the probability that a tag s_k does not exist in the library (false positive):

$$Q = -\log_{10}\prod_{i=1}^{N}\{1 - P(s_k|o_i)\} \quad (8)$$

For the results presented in this work, we required Q > 50.

Counting the Number of Exonic CLIP Tags Per Gene and Identifying FMRP and Hu Targets

We studied all annotated protein-coding genes and a set of candidate genes. For well annotated genes, we combined mouse RefSeq and UCSC known gene transcripts, which were grouped according to Entrez gene IDs (29,226 genes). Transcript or CDS length for each gene was calculated from the union of exons in these transcripts. CLIP tags overlapping with exons in these transcripts were considered to be exonic tags and counted to rank genes. In addition, to define candidate genes, we also clustered overlapping mouse mRNAs and ESTs, together with human RefSeq and UCSC known gene transcripts mapped to the mouse genome, using the program liftOver obtained from the UCSC Genome Browser (Rhead et al., 2010). We obtained 1,773 additional protein-coding genes annotated in the human, but not the mouse genome, resulting in a total of 30,999 genes used for this study.

We did not normalize tag number to transcript abundance, since FMRP is expressed primarily in neurons, while RNA from total brain polyribosomes includes transcripts from glial and other cell types.

To identify and rank FMRP targets, we used a nonparametric method motivated by meta analysis (Sokal and Rohlf, 1995) that considered both the total number of tags in seven different CLIP experiments, and also biological complexity (Chi et al., 2009; Darnell, 2010; Licatalosi et al., 2008) that measures the robustness of CLIP tags in different experiments.

Each experiment has a different number of tags, with significantly more tags obtained by Illumina sequencing. To weigh each experiment j (j = 1,…,7) properly, for each experiment, we first converted the number of exonic tags for each gene i (i = 1, …, N, N = 30,999) to a rank r_{ij} across all genes, from which a nominal single-experiment P-value was obtained by $p_{ij} = r_{ij}/N$.

P-values in all experiments were then combined by

$$\chi_i^2 = -2\sum_{j=1}^{7}\log(p_{ij})\,(i = 1, 2, \ldots, N) \quad (9)$$

which follows a χ^2 distribution with a degree of freedom 14, assuming the distribution of CLIP tags in different experiments is independent with each other. Overall P-values can then be derived from the χ^2 scores. False discovery rate (FDR) was estimated by the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

Mapping of CLIP Tags to Representative Transcripts

To study the distribution of CLIP tags along mature mRNA transcripts, we also mapped CLIP tags to a set of RefSeq transcripts to avoid the complication of exon junctions in direct mapping of reads to the mouse genome. For this purpose, we used a nonredundant set of 28,265 Refseq transcripts of protein-coding genes, one for each gene. For genes with multiple RefSeq transcripts, the longest transcript was chosen as the representative. The annotation of the 5′ UTR, CDS and 3′ UTR regions was tracked. In this case, both 454 and Illumina CLIP reads were aligned by BLAT.

The distribution of FMRP CLIP tags relative to start codons and stop codons were plotted using 762 of the 842 FMRP targets, which have RefSeq identifiers in mm9. The number of tags at each position is normalized by the number of transcripts that cover that position. The distribution of Hu tags relative to start codons and stop codons was plotted similarly using 842 Hu targets ranked by exonic tag numbers, among which 774 genes have representative RefSeq transcripts.

Gene Ontology Analysis

The enrichment of gene ontology (GO) terms in FMRP targets was analyzed using the online tool DAVID (Huang et al., 2009; Dennis et al., 2003). We obtained background genes either from our polyribosome exon array data or neuronal gene expression data ($\log_2$ intensity $\geq$ 7), as described above. DAVID reports a p-value, before and after multiple-test corrections, based on a modified hypergeometric test. p-values reported for this study was corrected using the Benjamini-Hochberg approach (Benjamini and Hochberg, 1995).

Comparison of FMRP Targets with Functionally Annotated Gene Sets

We compared the overlap of FMRP targets with diverse sets of genes with specific functional annotations, including groups of genes involved in cognition (PSP, NMDAR/MASC and mGluR, downloaded from http://www.genes2cognition.org), the pre-synaptic proteome (a total of 393 proteins identified both experimentally and through a literature/systems approach: see Croning et al., 2009, their Table S4 (Croning et al., 2009), 432 FMRP RIP-CHIP mRNA targets (available as Supplemental data in Brown et al., 2001 (Brown et al., 2001)), proteomic analysis (Aschrafi et al., 2005), the 80 FMRP targets identified by the APRA technique in Table 1 of Miyashiro

et al., 2003 (Miyashiro et al., 2003), the Simons SFARIGene database (Basu et al., 2009) (http://gene.sfari.org/) and the autism gene database, as of 7/18/2010; AGD (Matuszek and Talebizadeh, 2009) (http://wren.bcf.ku.edu/). For each compared gene set, we obtained the list of Entrez gene IDs to compare with FMRP targets. If the original gene set was defined in human, we obtained the mouse orthologous genes according to the Homologene database (Wheeler et al., 2006). In the case of autism susceptibility genes defined by CNVs, we obtained genes overlapping with each CNV by comparing their genomic coordinates. To focus on susceptibility genes with a higher confidence, we limited our analysis to relatively small CNVs (overlapping with $\leq$ 5 genes). Candidate genes were excluded from the compared datasets because many of them do not have mouse Entrez gene IDs. The overlap between each of the compared gene sets with FMRP targets were evaluated by Fisher's exact test.

To have a higher stringency for these comparisons, we also filtered genes according to their expression level according to brain polyribosome Affymetrix exon array data (or neuronal expression data (Cahoy et al., 2008)). Only genes with $\log_2$ intensity $\geq$ 7 were kept for analysis, no matter whether they were included in the list of FMRP or compared gene list.

Venn Diagram Generation

Venn diagrams depicting the degree of overlap of robust FMRP targets with various data sets in the literature were drawn to scale using the Venn diagram generator available online from the Bioinformatic and Research computing at the Whitehead Institute at http://jura.wi.mit.edu/bioc/tools/venn.php

Canonical Pathway Analysis Using Ingenuity Software

All 842 robust FMRP targets were uploaded to the core analysis module of the Ingenuity Pathways Analysis program (IPA 8.6-3003, Application Version 8.6, Build: 93815 (05/28/2010) Content Version 3003, Build: ing_lesath (4/28/2010) Ingenuity Systems, Inc.) by Entrez Gene ID. Of these, 788 were found to be ''network eligible'' and 731 ''functions/pathways/lists eligible'' and these were analyzed by the core analysis module using the Ingenuity knowledge base (genes only) as a reference set, 70 molecules per network analyzed, and otherwise default settings were used. Canonical pathways were analyzed three ways, first using all available pathways, second restricting analysis to intracellular and second messenger signaling pathways and lastly, restricting analysis to neurotransmitter and other neuronal signaling pathways. Fisher's exact test was used to calculate p-values and those pathways with p-values less than 0.05 were considered significant.

Cell Culture Ribosomal Run-Off Assays

Puromycin or hippuristanol-induced ribosome run-off assays were performed similarly to previously described experiments in our laboratory (Stefani et al., 2004). In brief, Neuro-2A cells, a mouse neuroblastoma line (catalog #CCL-131; American Type Culture Collection), were grown to 80% confluence in 15 cm dishes in DMEM containing 10% FBS, penicillin, streptomycin and supplemental L-glutamine, and were treated with the indicated concentrations of puromycin (Sigma; dissolved in water) for one hour followed by 100 μg/ml cycloheximide (CHX; Sigma C7698; dissolved in methanol) for 10 min. Hippuristanol (a generous gift from Dr. J. Pelletier, McGill University) was prepared in DMSO and added to the cells at 1 μM final concentration from a 1000X stock for the indicated times followed by 10 min in CHX. Cells were then washed twice with PBS, lysed in ice-cold polyribosome lysis buffer (20 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1X Complete protease inhibitor, EDTA-free (Roche), 100 ug/ml CHX, 40U/ml RNAsin (Promega) containing 1% NP-40 (Calbiochem)). A supernatant for polyribosome analysis was prepared by homogenization with 9 strokes at 900 rpm in a motor-driven glass-Teflon homogenizer, centrifugation at 5000xg, 10 min at 4°, and the supernatant was loaded onto 20%–50% w/w linear density gradients of sucrose in 10mM HEPES–KOH, pH 7.4, 150mM KCl or 150 mM NaCl, 5mM $MgCl_2$ and centrifuged at 40,000xg for 2 hr at 4°C in a Beckman Instruments (Fullerton, CA) SW41 rotor. Fractions of 0.72 ml volume were collected with continuous monitoring at 254 nm using an ISCO UA-6 UV detector (ISCO, Inc).

FXRP Knockdown and Puromycin Run-Off in N2A Cells

A mouse siGENOME ON-TARGETplus SMARTpool (Fisher Dharmacon) containing 4 siRNAs was used for each gene (*Fmr1*, *Fxr1* and *Fxr2*). N2A cells were transfected at 30% confluence in OptiMEM-I + GlutaMax medium (Invitrogen), with 11.7nM of each of the three oligo pools using Lipofectamine (Invitrogen) according to manufacturer's instructions. Medium was changed after 4-6 hr and knockdown was repeated the same way on the subsequent day. 15 hr after the second knockdown, cells were treated with 0.5 mg/ml puromycin (Sigma) for 1 hr, and then with 0.18 mg/ml cycloheximide for 20 min. A supernatant for polyribosome analysis was prepared as above.

IVT_{EBP} System and Ribosomal Run-Off Assays

Two- to three-week old wild-type mice (CD1, FVB or C57Bl6/J, both sexes) or paired littermates (*Fmr1* KO versus WT (FVB background) or I304N versus WT (C57Bl6/J background) (Zang et al., 2009), only males) were sacrificed by isoflurane anesthesia and decapitation. Postmitochondrial supernatants (S1) of cerebral cortex and cerebellum, dissected free from white matter, were prepared by homogenizing 2 mouse brains in 1 ml polyribosome lysis buffer (without addition of NP-40 or CHX) and yielded 300 μl of S1 following a 10 min, 2000xg spin at 4°C. ATP was added to 2 mM, 15 μl each of the –Met and –Leu amino acid mixtures (1 mM stocks, Promega nuclease-treated RRL kit L4960) and 150 μl of the nuclease-treated rabbit reticulocyte lysate (RRL, Promega L4960) were added to 300 μl of S1 and incubated at 30°C in the presence of puromycin (7.2 mg/ml) or hippuristanol (1 μM) for the

indicated times. 75 μl reactions were terminated by addition of 1 ml polysome lysis buffer including 1% NP-40 and 0.1 mg/ml CHX, on ice (10 min) followed by a 20,000 x g spin (10 min, 4°C) and S2 was applied to 20%–50% sucrose gradients and processed as described for cells. Where indicated, in vitro transcribed RNAs (1.0 uM final concentration; unlabeled) were added to the S1 lysate (after heating RNA to 75 degrees for 10 min and bench cooling for 5 min in 1X SBB buffer (Selex binding buffer, 50 mM Tris acetate, pH 7.7, 200 mM potassium acetate, 5 mM Mg-acetate) as previously described for filter binding assays (Darnell et al., 2009) at the start of the incubation.

In one set of experiments, kcRNA or mutant kcRNA was added after puromycin run-off, as follows. Brains from three WT FVB mice aged P14 were dissected of white matter and homogenized together in 750 μl polyribosome lysis buffer (without addition of NP-40 or CHX). Polyribosome run-off was conducted in the presence of 16.4 uM puromycin as above, for 20 min at 30°C, and then placed on ice water. 1.2 mM CHX and 0.2 mM anisomycin were added for 10 min on ice water, followed by 1 uM kcRNA or 1 uM mutant kcRNA. Samples were incubated on ice water for 20 min, then diluted in 2.5 volumes gradient buffer containing protease inhibitors, RNAsin Plus, 1.2 mM CHX, 0.2 mM anisomycin and 1% NP-40. Samples were incubated on ice 10 min, then loaded onto 20%–50% (w/w) sucrose gradients.

To monitor nascent protein synthesis in the IVT_{EBP} system, puromycin was omitted from the reaction, 30 μl –Met amino acid stock was used instead of a combination of amino acid pools, and 60 μl ^{35}S-methionine (Perkin Elmer NEG-009T, specific activity 1175 Ci/mmol) was added per 300 μl of S1. For the time course (Figure S3B-C) 150 μl reactions were performed with either CHX, hippuristanol at 10 μM, or with an equivalent volume of DMSO and 10 μl aliquots were removed from the tubes into 0.5 ml of IP wash buffer (10 mM HEPES–KOH, pH 7.4, 150 mM KCl or 150 mM NaCl, 5 mM $MgCl_2$ plus 0.1% SDS, 25 mM EDTA, 1% NP-40) to each to halt translation at the indicated time points. 12 μl was separated by 16% SDS-PAGE, fixed in 25% isopropanol, 10% acetic acid for 30 min and soaked in Amplify (GE Healthcare) for 30 min prior to drying and exposure to MR film (Kodak) or PhosphorImager.

EDTA Treatment of Polyribosomes in the IVT_{EBP} Ribosomal Run-Off Assay

FVB P15 brain lysate was prepared for the IVT_{EBP} assay, and run-off performed in the presence of ATP, amino acids, and RRL as described above. Samples were split into 3 aliquots and treated with either 1 uM kcRNA, 1 uM kcRNA + 30 mM EDTA, or 1 uM $kcRNA_{C50G}$ mutant. Puromycin was then added and each reaction was allowed to run off for 20 min at 30°C. Reactions were stopped by the addition of 1 ml of lysis buffer containing CHX and 1% NP-40. Samples were incubated on ice for 10 min and then loaded onto sucrose gradients for analysis.

In Vitro Transcription of Decoy RNA Ligands

In vitro transcription of unlabeled kcRNA and $kcRNA_{C50G}$ was performed as previously described (Darnell et al., 2009; Zang et al., 2009). Labeled RNA was treated with 3 units of RQ1 DNase and gel purified on 8% urea-polyacrylamide gels followed by excision and precipitation. Quality was checked by end labeling with T4PNK and γ-^{32}P-ATP and running on 8% urea-polyacrylamide gels, drying, and exposure to Kodak MR film.

Immunoprecipitation Analyses from ^{35}S-Methionine Labeled Proteins in IVT_{EBP} System

Proteins were labeled in the IVT_{EBP} system using 4 CD1 mice (P22) for brain polyribosome lysate preparation to yield 0.6 ml of S1. The complete IVT reaction (including 1 μM hippuristanol) was divided into 13 × 75 μl aliquots and kcRNA added to 6 replicates and $kcRNA_{C50G}$ to the other 6 replicates and incubated at 30°C for 15 min. One aliquot received 0.1 mg/ml CHX as a control. The reactions were stopped by 5 min incubation on ice and addition of 0.5 ml IP wash buffer (10 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1X Complete EDTA-free protease inhibitor cocktail (Roche), 1% NP-40, 0.1% SDS). Aliquots of each reaction (100-200 μl) were precleared with 25 μl of rabbit anti-mouse IgG (Jackson Immunochemicals #315-005-008) loaded protein A Dynabeads (Dynal/Invitrogen) for 1 hr at 4°C, and the supernatant was IPed with protein A Dynabeads loaded with either anti-Camk2a (Stressgen KAM-CA002) for 2 hr, anti-Lingo1 (Abcam 23631) overnight, or anti-Pabpc1 (Abcam 21060) overnight. All steps were performed at 4°C. Captured beads were washed 4 X with 1 ml IP wash buffer and beads eluted with reducing LDS sample buffer (Invitrogen) at 70°C, for 10 min with shaking. Proteins were separated on 4%–12% Novex NuPAGE Bis-Tris midigels in MOPS running buffer and gels fixed, Amplified, dried and PhosphorImaged as above. Specific bands corresponding to IPed targets were quantified using Quantity One software and signal from the identical region in the CHX-treated sample lane subtracted from each band. Each specific band was then normalized to another nonspecific band in the same lane of the gel.

To determine total protein labeling in the IVT_{EBP} system proteins were labeled using 2 CD1 mice (P22) for brain polyribosome lysate preparation to yield 0.3 ml of S1. The complete IVT reaction was divided into 7 × 75 μl aliquots and kcRNA added to 3 replicates and $kcRNA_{C50G}$ to the other 3 replicates and incubated at 30°C for 15 min. One aliquot received 0.1 mg/ml CHX as a control. The reactions were stopped by 5 min incubation on ice and addition of 0.5 ml IP wash buffer (10 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1X Complete EDTA-free protease inhibitor cocktail (Roche), 1% NP-40, 0.1% SDS). 10 μl aliquots were mixed with loading buffer and run on 16% SDS-PAGE, fixed, soaked in Amplify, dried and quantified by PhosphorImaging. Linearity of detection of signal in the appropriate range was determined by loading increasing volumes of sample, PhosphorImaging and plotting quantified signal against ul loaded (Figure S6A). Labeling of three different size ranges of products (indicated with brackets in Figure S6B) was quantified using Quantity One software after subtracting an identical area in the CHX lane and normalizing all values as a % of the first $kcRNA_{C50G}$ sample on the gel (Figure S6C).

Microarray Analysis of Differential mRNA Distribution in WT versus Fmr1 KO Polyribosomes

Polyribosomes from cerebral cortices of P7 male WT and paired *Fmr1* KO littermate mice (FVB background) were prepared as previously described (Stefani et al., 2004). Two mice of each genotype were pooled per polysome gradient. Six biological replicates from each genotype were prepared. 20% of the total S2 was reserved for total RNA "input" before application to the gradients. Following fractionation 15% of each fraction was analyzed by Western blot and RNA was extracted from the remaining 85% of each fraction using Trizol LS as described below. RNA from fractions 10-13, corresponding to association with 6 or more ribosomes, was pooled as polyribosomal RNA. Both total and polyribosomal RNAs were labeled by standard methods and used to interrogate Affymetrix Mouse 430 2.0 GeneChips.

Microarray Data Processing

The Mouse Genome 430 microarray data were processed to estimate gene expression level using gcRMA in Bioconductor (Wu et al., 2004). Differential expression between wild-type or FMRP knockout brains was evaluated by the naive Bayes method, using total RNA level or normalized polysome RNA level (Smyth, 2004).

RNA Preparation and qRT-PCR Analysis

RNA from polyribosome fractions was extracted using Trizol LS Reagent according to manufacturer's instructions (Invitrogen) and then back-extracted in chloroform:isoamyl alcohol (49:1) and spun at 10,000 × g for 15 min. The aqueous phase was precipitated with 15 μg Glycoblue (Ambion) and 1 volume isopropanol. RNA was pelleted at 20,000 x g for 10min at 4°C, washed twice with cold 75% ethanol and resuspended in water. Samples were digested with RQ1 DNase (Promega) for 1 hr at 37°C, extracted with phenol-chloroform, back-extracted with chloroform:isoamyl alcohol, precipitated with Glycoblue in 2 volumes of 1:1 ethanol:isopropanol and washed as before. cDNA was prepared with equal volumes of RNA from each fraction, using random hexamers (Roche) and Superscript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. cDNAs were amplified by QPCR using iTaq SYBR Green Supermix with ROX (Biorad) and 200 nM primers (sequences included in following section). qPCR was performed on an iQ5 Multicolor Real-Time PCR Detection System (Biorad) using a 58°C annealing temperature. Relative RNA levels were calculated using a Ct threshold of 100 and the formula $2^{(40-Ct)}$. Normalization to an internal control was not necessary. For each gene, polyribosome fractions were plotted as percentages of the total amount of the gene on the gradient (obtained by summing values of individual fractions). Error bars refer to technical replicates of qPCR measurements within single experiments.

Primer Sets Used for qRT-PCR

Bsn Bassoon (NM_007567)

F: GGAGCACTCCTCTACGTTGC; R: GCTGCTGCTTTGCTTCTTCT

Kif1a Kinesin 1A (NM_008440)

F: ACCAGAGTGGGCAGAGAAGA; R: TGTGTCTGCTCCTTCACAGG

Adcy1 Adenylate cyclase 1 (NM_009622)

F: CTTACCAGGGAAGGTCCACA; R: GGCTGGTTTGATATCCGAGA

Cyfip2 Cytoplasmic FMRP-interacting protein 2 (NM_133769)

F: CGGTCTTGGACGAGCTAAAG; R: CACTGGGTGATCCTGTTGTG

Mtap1b Map1b (NM_008634)

F: CGGACAGTGCTTTGAGAACA; R: GTGGTGCTTAGGAGCTCACC

Nisch Nischarin (NM_022656)

F: GACAGGTGGCCTCTGATGAT; R: CTGAGGCGCTGGATAAAGTC

Bai2 Brain-specific angiogenesis inhibitor 2 (NM_173071)

F: GGGACCTGCTCTTCTCTGTG; R: AATCCACCATGAAGCTCACC

Atp1a3 ATPase, Na^+/K+ transporting, alpha 3 (NM_144921.1)

F: CCATTGTCACTGGTGTGGAG; R: CAATGCAGAGGATGGTGATG

Bat2 HLA-B associated transcript 2 (NM_020027)

F: CCTACCCCAACACCAGAGAA; R: TGGTTCAGGACCCTCTTTTG

Pde2a Phosphodiesterase 2a (NM_001008548)

F: GGGAGTCCAGACTGGTGTGT; R: CAGGGGTATGACCAGCACTT

Camk2a Ca/Calmodulin dep. kinase IIa (NM_09792.3)

F: TCTGAGAGCACCAACACCAC; R: CCATTGCTTATGGCTTCGAT

Grik5 Kainate receptor 5, KA5 (NM_008168.2)

F: AGTACGAGACCACGGACACC; R: CGAAGCGAAGGTACTGAAGG

Grin2b NMDAR subunit 2B (NM_008171)

F: TGCGCTCTCCCTTAATCTGT; R: GCCAACACCAACCAGAACTT

Lingo1 Lingo1 (NM_181074.4)

F: CCTGAGGATCCATCCAGAAA; R: AGCCTGTAGCAGAGCCTGAC

Dlg4 PSD-95 (NM_007864.2)

F: AACAGAGGGGGAGATGGAGT; R: CAAACAGGATGCTGTCGTTG

Dlgap3 SAPAP3 (NM_198618)

F: TCAGATGGTAGCCCCAAGAC; R: TGTAGCCAGGGATGGAACTC

Grin2a NMDAR subunit 2A (NM_008170)

F: GGTTCTGCATCGACATCCTT; R: GAACGCTCCTCATTGATGGT

Pctk1 PCTAIRE protein kinase 1 (NM_011049.4)

F: GCTAGACAAGCTGGGTGAGG; R: AGTACAGGGTGCCCCTTCTT

Ndrg4 NDRG family member 4 (NM_145602.2)

F: CAGCCATCCTCACCTACCAT; R: TGGCACACCACAAAGTGTTT

Gnb1 G protein, beta polypeptide 1 (NM_008142.4)

F: GCGGGACACACAGGTTATCT; R: CAGTCTGGTGTCAGGAGCAA

Ttyh3 Tweety homolog 3 (NM_175274.4)

F: GGAGGTGCTAGCTGAACAGG; R: AGACCCCAACCAAGATACCC

Arc Arg3.1/Arc (NM_018790)

F: GAGAGCTGAAAGGGTTGCAC; R: GCCTTGATGGACTTCTTCCA

Pabpc1 PABP (NM_008774.3)

F: GAGACCAGCTTCCTCACAGG; R: GGACTCCCGCAGCATATTTA

Gria1 AMPAR subunit GluR1 (NM_008165.3)

F: ACCACTACATCCTCGCCAAC; R: TCACTTGTCCTCCACTGCTG

Hyou1 Hypoxia upregulated 1 (NM_021395.4)

F: TACCAAACTTGGCAACACCA; R: GGCTCTCCTCTTCCTCCTGT

Clcn6 Chloride channel 6 (NM_011929.2)

F: CTGGAGGTGTTGGAGACCAT; R: ACGTTTGAACCACTCCGAAC

Sae1 SUMO1 activating enzyme subunit 1 (NM_019748)

F: CTGCTGCAGATACGGAATGA; R: CCAACCACAGCACATACTGG

Hprt1 Hypoxanthine phosphoribosyltrans. 1 (NM_013556)

F: AAGCTTGCTGGTGAAAAGGA; R: TTGCGCTCATCTTAGGCTTT

Eif3a Eukaryotic initiation factor 3a (NM_010123.3)

F: ACTCCCTGGGTCAAGTTCCT; R: CACATAGCTTGCGGAACTCA

Tcerg1 Transcription elongation regulator 1 (NM_001039474.1)

F: TTCGAGAACGGGAAAGAGAA; R: CCGGAGGGTCCTCCTAGTAT

Npepps Aminopeptidase puromycin sensitive (NM_008942.2)

F: CAAAAGAATGCTGCCACAGA; R: GGTCCACTGGCACAAAACTT

Gria2 AMPAR subunit GluR2 (NM_001039195)

F: AACGGCGTGTAATCCTTGAC; R: CACCAGGGAGTCGTCGTAGT

Vldlr Very low density lipoprotein receptor (NM_013703.2)

F: ACGGCCAGTGTGTTCCTAAC; R: ATTTTCACCATCGCATCTCC

Canx Calnexin (NM__001110499.1)

F: GCCTGAAGATTGGGATGAAA; R: CAATCCTCTGGCTTCTCTGC

Glrb Glycine receptor, beta (NM__010298.5)

F: TGTGGTTCTCTCCTGGCTCT; R: CAGCGCCTTCACATAAGACA

Slc35f1 Solute carrier family 35, member F1 (NM_178675)

F: CGGTCAACCTTTCTCTGCTC; R: GGGGATCCTGGGCTATGTAT

St8sia3 ST8 a-N-Ac-neuraminide a-2, 8-sialyltransf. 3 (NM_009182.2)

F: CGGACAGCGTTTTTACATCA; R: AGCAGGGACCGGAAGTTATT

Atp6ap2 ATPase, H^+ transp., lysosomal acc. prot.2 (NM_27439.3)

F: TTTGGATGAACTTGGGAAGC; R: CACAAGGGATGTGTCGAATG

Tmem65 Transmembrane protein 65 (NM_175212.4)

F: CATCTACAGCCTGCACTCCA; R: GCGTTATCCAAAAAGCCAAA

Estimation of Ribosomes/mRNA Per Sucrose Gradient Fraction

A representative gradient from a CHX-treated sample was used to quantify the number of ribosomes per gradient fraction. Individual ribosome peaks on the A_{254} trace were resolved between fractions 4 and 9, allowing polyribosome size to be directly counted up to 10 ribosomes. Polyribosome sizes in fractions 10-16 were extrapolated by fitting a polynomial trend line to the resolved data in fractions 4-9 ($y = 0.125x^2 + 0.025x - 0.8$). To verify that the sucrose gradient was linear, the concentration of sucrose in each fraction of a blank gradient was measured using a sucrose refractometer (Hanna Instruments).

Selection of FMRP Targets and Nontargets for Analysis

Genes were selected for validation as FMRP targets on the basis of total number of CLIP tags. Because many top targets have long coding sequences compared to nontargets, several targets of moderate CDS length were included. Nontargets were required to have few CLIP tags, moderate to high representation in polyribosome fractions (measured by Affymetrix Exon Array) and strong

neuronal expression (based on the neuronal transcriptome previously described (Cahoy et al., 2008)). Special effort was made to include nontargets of long CDS length.

Calculation of Ribosome Retention Score and Ribosome Load

In order to quantify the FMRP-dependent effect on ribosome stalling we devised a means of estimating ribosome occupancy on mRNA in polyribosome gradient fractions, and weighting the differences in polyribosome profiles from WT and *Fmr1* KO fractions for this ribosome occupancy. By counting the number of ribosomes in each fraction from A_{254} traces and extrapolating, the average number of mRNA-associated ribosomes in each gradient fraction can be estimated (Figure 6A), based on linearity of the sucrose gradients). Multiplying the percentage of mRNA in each fraction by the number of ribosomes in that fraction and summing over the gradient puts a value on total ribosome occupancy (''ribosome load'') under any one condition. The difference between this value of stalled ribosomes from WT and the corresponding FMRP loss-of-function model value (*Fmr1* KO, I304N, or kcRNA-treated) after puromycin run-off yields an FMRP-dependent ribosome retention score (Table S6, RRS).

Electron Microscopic Analysis of Sucrose Gradient Fractions Containing Stalled Polyribosomes

Negative staining with uranyl acetate was performed by the Rockefeller University Electron Microscopy Resource Center as follows: 10 ul of fraction 9-10 from a WT puromycin-treated IVT_{EBP} experiment sucrose gradient was deposited on a glow discharged, carbon-coated grid (EMS Cat# CF400-Cu-50) for 30 s to 1 min. After washing the sample quickly by passing over two drops of dd-H_2O, the grid was then treated with 2 drops of 2% uranyl acetate (Fisher Cat#79853) for a total of 30 s. Excess stain was removed by touching the edge of the grid carefully to a filter paper wedge. After air drying the grid was viewed with a TecnaiSpiritBT Transmission Electron Microscope (FEI) at 80 KV and pictures were taken with Gatan 895 ULTRASCAN Digital Camera.

Immunogold-electron microscopy to detect polyribosomes by localization of EGFP-tagged ribosomal protein L10a was performed following 20%–50% sucrose gradient purification of cerebellar extracts, prepared as described above, from Bac transgenic Purkinje cell-specific *Pcp2*-promoter driven EGFP-tagged rpL10a adult male mice (provided by Dr. Nat Heintz, Rockefeller University). Sucrose gradient fraction 9 or 10 was fixed in suspension using concentrated paraformaldehyde (PF) (16%) to reach a final concentration of 4% PF on ice. 10 ul of sucrose fraction 9 or 10 was deposited on a glow discharged and carbon-coated grid for 1 min and fixed again in 0.08% glutaraldehyde for 10 min at RT. After PBS washes and blocking in 1% BSA/PBS the polyribosomes were incubated first with anti-GFP antibody (Abcam #ab6556, 1:1000 dilution) and then with 6nm or 12nm colloidal gold conjugated to goat anti-rabbit IgG (H^+L) for 30 min. The grids were washed and negatively stained with 2% uranyl acetate (Fisher Cat#79853) for a total of 30 s. Excess stain was removed by touching the edge of the grid carefully to a filter paper wedge. After air drying the grid was viewed with a TecnaiSpiritBT Transmission Electron Microscope (FEI) at 80 KV and pictures were taken with Gatan 895 ULTRASCAN Digital Camera.

In experiments using transfected EGFP-FMRP in cultured cells, 20 ug of pEGFP-C1 (negative control expressing only EGFP) or pEGFP-iso7-hFMRP (Darnell et al., 2005b) was transfected using FuGene 6 into 6 100 mm tissue culture plates of HEK293T cells at 30% confluence and allowed to express for 48 hr. Plates were treated with 1 mM puromycin or vehicle alone for 1 hr in the incubator, followed by 0.1 mg/ml cycloheximide for 20 min Plates were washed twice with warm PBS and post mitochondrial cell lysates were prepared and fractionated on 20%–50% (w/w) linear sucrose gradients as described above. 10 ul of each fraction number 10 was fixed in 4% PF at 4°C overnight. 10 ul was deposited on a glow discharged and carbon-coated grid for 1 min and fixed again in 0.095% glutaraldehyde for 10 min at RT. The remainder of the protocol was performed as in the previous paragraph with 12 nm gold colloidal gold conjugated to goat anti-rabbit IgG (H^+L).

Micrococcal Nuclease Sensitivity Assays

Following standard polyribosome fractionation of mouse brain in the presence of CHX, as described above, fractions containing FMRP were pooled, diluted 1:1 with gradient buffer (10 mM HEPES-KOH pH 7.4, 150 mM NaCl, and 5 mM $MgCl_2$) and $CaCl_2$ was added to a final concentration of 1 mM from an RNase-free 100 mM stock. Samples were treated with the indicated concentration of micrococcal nuclease (0, 100, 500 or 1000 U/ml final concentration) for 20 min at room temperature with end-over-end mixing. Reactions were stopped by the addition of 2 mM EGTA and placed on ice. For high speed sedimentation assays (e.g., Figure 7D) 1 ml samples were spun in a TLA120.2 rotor in a Beckman Optima tabletop centrifuge for 2 hr at 280,000 x g. Supernatants were carefully removed and equal volumes of supernatant and input to the spin (saved before centrifugation) were TCA precipitated and analyzed by Western blot for rpP0, PABP, and FMRP. Pellets were washed twice with PBS and resuspended, and restored to the original volume (1 ml). Note that in these assays the 1 ml treated with MN had a sucrose concentration of approximately 17.5% based on determination of the sucrose concentration in pooled fractions with a refractometer and accounting for the 1:1 dilution. For analysis by sucrose gradient fractionation samples were loaded on linear 20%–50% sucrose gradients as described above. TCA precipitation of samples of each fraction and gel eletrophoresis was followed by Western blot for FMRP (ab17722), rpP0 and PABP and quantitation of the gels by Quantity One software is presented instead of the gels for higher MN concentrations to demonstrate that no additional size decrease in the FMRP complex is observed as a function of MN concentration.

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